

Antibacterial Activity of Selected Plant Extracts on Methicillin Resistant *Staphylococcus aureus*

Lubna Abdallah¹ and Shurooq Ismail²

Biology and Biotechnology Department, Faculty of Science, An Najah National University
alubna@najah.edu¹, Shurooq.ismail@najah.edu²

Abstract

*Based on the importance of herbal plants in medicine, this study was conducted to evaluate the antibacterial efficacy of aqueous, ethanol and methanol extracts of *Thymbra spicata*, *Nepeta curviflora*, and *Verbascum fruticosum* against two clinical isolates (I and II) of the gram positive methicillin resistant *Staphylococcus aureus* (MRSA). Agar well diffusion method was performed to examine the antibacterial activity of all studied plant extracts. Micro-broth dilution method was used to measure the minimum inhibitory concentration (MIC) then the minimum bacteriocidal concentration (MBC) for all active extracts. The examined isolates were susceptible to the prepared plant extracts. Ethanol and methanol extracts of *T. spicata* were the most efficient extracts. Ethanol and methanol extracts for all plants in this study showed higher antibacterial activity compared to aqueous extracts. Among the studied plant extracts, *T. spicata* extracts showed the best antibacterial effect for MRSA as they act as bacteriocidal agents at concentration range (0.781-6.25) mg/ml. Fractionation and characterization of *T. spicata* active components may enhance the pharmaceutical industry of new drugs against MRSA.*

Keywords: *Antibacterial, Methicillin Resistant Staphylococcus aureus, Thymbra spicata, Nepeta curviflora, Verbascum fruticosum.*

Introduction

During the last decade, the use of traditional medicine has expanded globally and it is gaining more popularity. Plants are used medicinally in different countries and considered as a source of many potent and powerful drugs (Srivastava et al., 1996). Medicinal plants represent a rich source of antimicrobial agents, as they protect themselves from different pathogens via the production of different chemicals including tannins, flavonoids, terpenoids and alkaloids (Obeidat et al., 2012). Medicinal plants are highly used in Palestine as utilization of complementary and alternative medicine is very common (Sawalha, 2007). In Palestine, there are several studies concerning the different biological activities of many plant extracts. These plant extracts were used as antibacterial, antitumor, antifungal and antioxidant agents (Ali-Shtayeh et al., 1997; Ali-Shtayeh and Abu Ghdeib, 1998a; Ali-Shtayeh et al., 1998b; Abu-Shanab et al., 2004; Abu-Shanab et al., 2006; Husein et al., 2010; Omar et al., 2013; Fares et al., 2013).

Staphylococcus aureus is a gram-positive bacterium which is one of the leading causes of human infections of the skin, soft tissues, bones and joints, abscesses, as well as normal heart valves (Karlowsky et al., 2003). Methicillin Resistant *Staphylococcus aureus* (MRSA) was first recognized in the 1960s (Barber, 1961). It is a multiresistant strain that has been documented worldwide showing rising resistance to different classes of antimicrobials (Ness, 2010). The main aim of this study is to find a natural antibacterial agent which is extracted from plant species that are found in Palestine. The first studied plant is *Thymbra spicata* L. from Lamiaceae family. *T. spicata* contains essential oils that made it an important antibacterial spice for the treatment of asthma and bronchitis in the traditional medicine (Daneshaver et al., 2009). There are several studies on the antimicrobial activity of *T. spicata* essential oils (Tümen et al., 1994; Kilic, 2006; Akin et al., 2010; Markovic et al., 2011). The second plant species in this study is *Nepeta curviflora* Boiss which belongs to the Lamiaceae family. There are many studies focusing on the essential oils composing this genus (Baser et al, 1993; Moghaddam et al., 1996; Kokdil et al., 1997; Baser et al., 1998; Rustaiyan et al., 2000; Thappa et al., 2001; Ghannadi et al., 2003; Dabiriand Sefidkon, 2003). Other studies about different *Nepeta* species have showed antibacterial activity against several bacterial strains (Sonboli et al., 2004; Alim et al., 2009; Adiguzel et al., 2009; Bisht et al., 2010). The third plant in this study is *Verbascum fruticosum* Post, which is a member of *Verbascum* genus. *Verbascum* is commonly known as mullein, which is a widespread genus of the Scrophulariaceae family. Some *Verbascum* species have been used for their medicinal effects in Turkey (Dulgeret al., 2005b). Turker and Camper (2002)

showed that *K. pneumoniae* and *S. aureus* were sensitive to the Mullein, which may explain why Mullein is used in folk medicine to treat respiratory disorders. In addition to that, several *Verbascum* species showed antibacterial activity against *S. aureus* (Dulger and Ugurlu, 2005a). In this study, our work was concentrated on the discovery of effective plant extracts that act as powerful antibacterial medicines against MRSA clinical isolates. The extracts in this study were prepared using water, ethanol and methanol as solvents for crude extract of the three mentioned herbal species.

Hypothesis

Herbal plants are a good source of antibacterial compounds that could be used in the treatment of multi-drug resistant bacteria like MRSA.

Methodology

Antibiotic Screening Assay

The in vitro antibacterial activities of the plant extracts were evaluated against gram positive methicillin resistant *Staphylococcus aureus* (MRSA). The two clinical isolates (I and II) were identified by Ghaleb Adwan Department of Biology & Biotechnology, An-Najah National University, Palestine. The susceptibility of these clinical isolates was tested on iso sensitivity test agar plates using seventeen antibiotics which are: Methicillin, Penicillin G, Oxacillin, Clindamycin, Vancomycin, Tetracycline, Gentamicin, Erythromycin, Cephalothin, Colistin, Ciprofloxacin, Nitrofurantoin, teicoplanin, Trimethoprim/Sulfamethoxazole, Ceftazidime/clavulanic acid, Aztreonam and Chloramphenicol.

Plant Materials

The three studied plant species (*T. spicata*, *N. curviflora* and *V. fruticosum*) were collected from different locations in West Bank, Palestine. The plants species were identified by Ghadeer Omar, Department of Biology & Biotechnology, An-Najah National University, Palestine. Representative plant specimens of the studied plant species were collected, pressed till drying, treated chemically, mounted on herbarium sheets and provided with voucher numbers, after they are deposited at An-Najah National University herbarium. The plant materials for the antibacterial assay were washed, air-dried, ground into powder using a grinder and stored at a room temperature until they were used.

Aqueous Extraction of Plants

Ten grams of each plant powder were soaked in 100 ml sterile boiled distilled water for one week with interval shaking. Then the mixtures were centrifuged for 5 min at 5000 rpm. The supernatants were evaporated by freeze-drying. The extracted powder of each of the plant species under investigation was dissolved in distilled water to a final concentration equal to 100 mg/ml.

Alcohol extraction

Ten grams of each plant powder were soaked in 100 ml of (70 %) ethanol or methanol for one week with interval shaking. Then the same steps of aqueous extraction were repeated, except that (10%) dimethyl sulfoxide (DMSO) was used as a solvent instead of the distilled water.

Antibacterial Activity Assay

The antibacterial activity of the investigated plant extracts were determined by well diffusion method (NCCLS, 1999). The tested bacteria were grown overnight on nutrient agar plates. Broth turbidity was adjusted to 0.5 McFarland (1.5×10^8 CFU). Then each bacterium (from two isolates) was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately (60°C) each time to ensure an even distribution of the inoculum. As a final step, the rim of the agar was also swabbed. After 10 minutes, (6 mm) wells were bored in the agar. Each plant extract under study was checked for the antibacterial activity by introducing (25 μl) of a (100 mg/ml) concentration of the plant extract into each well. The plates were allowed to stand at room temperature for (30 min) for extract to diffuse into the agar and then they were incubated at (37°C) for (18 h). Then the plates were examined for bacterial growth inhibition by measuring the inhibition zone diameter (IZD) to the nearest mm. The test was performed in duplicates. Antibiotic Tetracyclin was used as a positive control and sterilized distilled water and (10 %) DMSO was used as a negative control.

Minimum inhibitory concentration (MIC)

All active plant extracts were tested for their minimum inhibitory concentration by micro-broth dilution method (NCCLS, 2000). The prepared extract was serially diluted two fold in nutrient broth medium. Duplicates of each dilution (50.0, 25.0, 12.5, 6.25, 3.125, 1.563, 0.781 0.391, 0.195 and 0.098 mg/ml) were inoculated with (1 μl of 1×10^7 CFU/ml). The last two duplicate wells were not inoculated. Then the inoculated microtiter plates were incubated at (37°C) for

18h. The lowest extract concentration (highest dilution) that inhibited the growth of the tested microorganisms was considered as MIC.

Minimum bactericidal concentration (MBC)

In this technique, the contents of the wells resulting from MIC was streaked using a sterile cotton swabs on agar plate free of antibacterial agents and incubated at (37°C) for (18) h. The lowest concentration of the extract, which showed no bacterial growth, was considered as MBC.

Results

Both clinical isolates of the studied bacteria showed resistance to half of the screened antibiotics, especially methicillin, oxacillin and vancomycin (Table 1).

Table 1. Susceptibility of MRSA* isolates I and II to 17 antibiotics

Antibiotic		MRSA I Inducibility	MRSA II Inducibility
Methicillin	MET	R	R
Penicillin (10)	P	R	R
Oxacillin (1)	OX	R	R
Clindamycin	DA	S	S
Vancomycin (30)	VA	R	R
Tetracyclin (30)	TE	S	S
Gentamicin (10)	GM	S	S
Erythromycin (15)	E	S	R
Ciprofloxacin (5)	CIP	S	R
Trimethoprim/Sulfamethoxazole (25)	SXT	R	S
Colistin (10)	CT	R	R
Nitrofurantoin (300)	F	S	S
Cephalothin (30)	KF	S	S
teicoplanin (30)	TEC	S	S
Aztreonam (30)	ATM	R	R
Ceftazidime/clavulanic acid (40)	CZC	R	R
Chloramphenicol (30)	C	S	S

*MRSA was tested on iso sensitivity test agar, R: resistant; S: sensitive

Agar well diffusion method of aqueous, ethanol, and methanol plant extracts in our study showed an antibacterial activity against the two isolates of MRSA. Table 2 illustrates that the ethanol and methanol extracts of *T. spicata* produced the highest antibacterial activities compared to the other investigated plant extracts. They produced (20mm) inhibition zone against both isolates of MRSA. The same antibacterial effects of ethanol and methanol extracts of *N. curviflora* were also recorded. The inhibition zones that resulted from *N. curviflora* on MRSA I and II were smaller than those resulted from *T. Spicata*. Unfortunately, water extract of *V. fruticosum* had no effect on both isolates at the examined concentration (100 mg/ml). On the other hand, *V. fruticosum* alcoholic extracts showed antibacterial effect.

Table 2. The Antibacterial activity of the tested plant extracts against the two clinical isolates of MRSA

Plant species	Extract Type	IZD*	
		MRSA I	MRSA II
<i>N. curviflora</i>	Ethanol	17	12
	Methanol	14	10
	Water	10	11
<i>T. spicata</i>	Ethanol	20	20
	Methanol	20	20
	Water	14	15
<i>V. fruticosum</i>	Ethanol	10	10
	Methanol	13	11
	Water	0	0
Positive control	Tetracycline (30)	27	27

* (IZD) diameter of inhibition zones (mm) including the diameter of the well (6 mm); values are the mean of two duplicates; (0) not active

The antimicrobial activity of the extracts under study was quantitatively assessed by determining the MIC concentration for each bioactive extract against two bacterial isolates. Figure 1 and 2 clearly showed that both alcoholic extracts were more effective than aqueous extract for all plants in this conducted experiment. The lowest MIC values were recorded for all extracts prepared from *T. spicata* particularly on MRSA II.

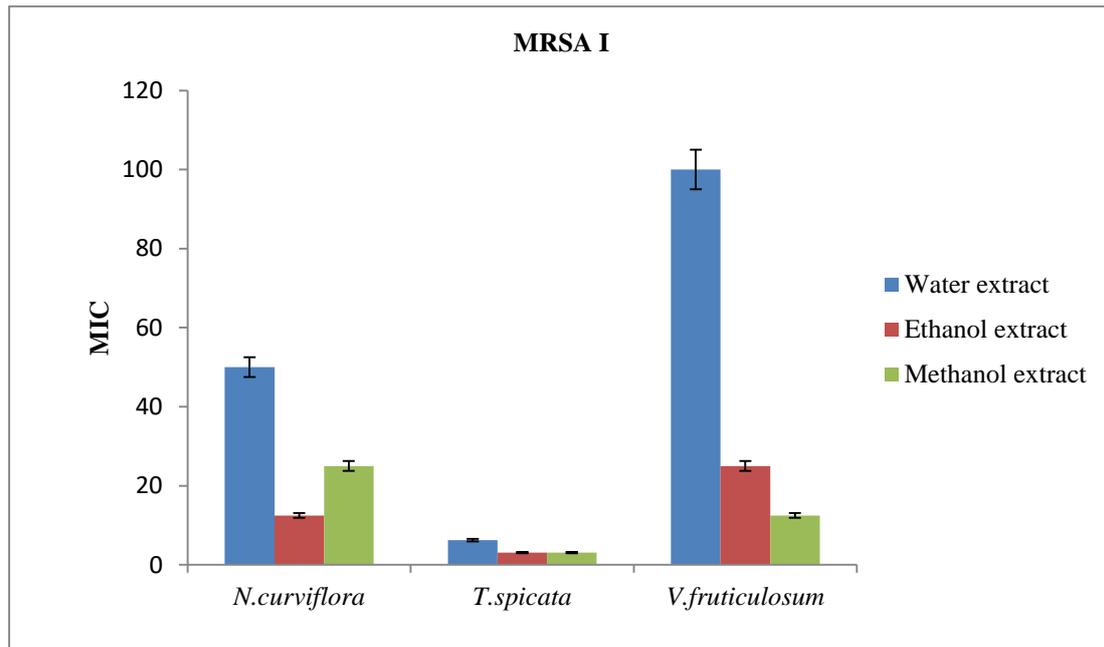


Figure 1. Antibacterial activity of *T. spicata*, *N. Curviflora* and *V. fruticulosum* against MRSA I using micro-broth dilution method; (MIC) minimum inhibitory concentration (mg/ml)

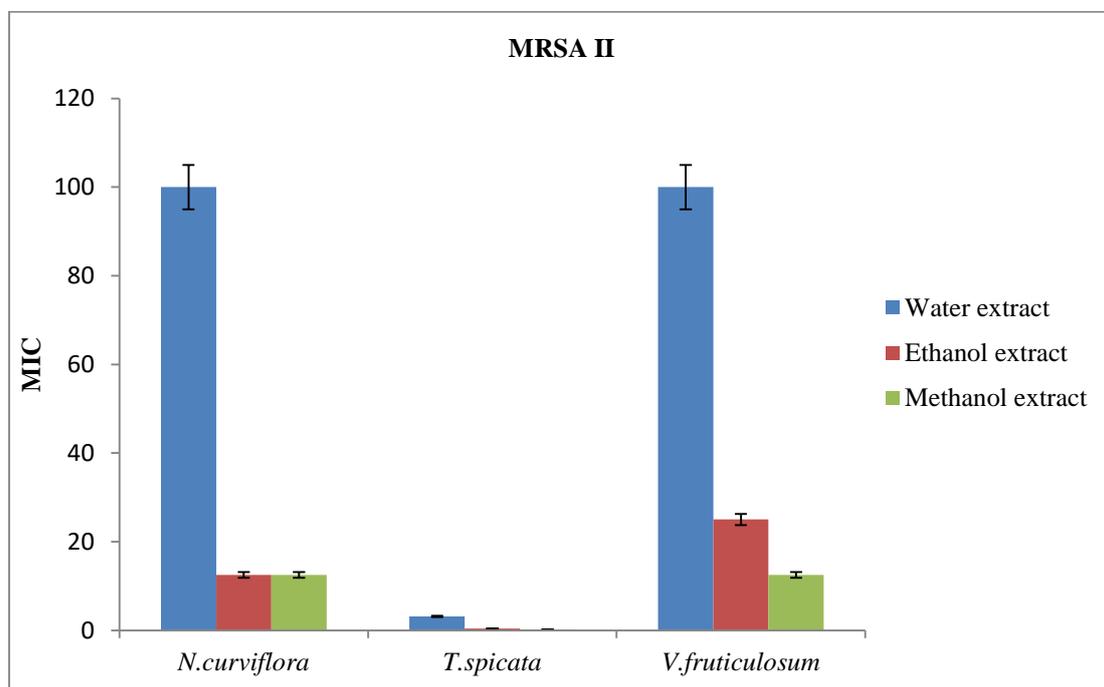


Figure 2. Antibacterial activity of *T. spicata*, *N. Curviflora* and *V. fruticulosum* against MRSA II using micro-broth dilution method; (MIC) minimum inhibitory concentration (mg/ml).

The minimum bacteriocidal concentrations (MBC) for all extracts that were given inhibitory effect in our research were determined. Figure 3 and 4 showed that all *T. spicata* extracts were the best as they were killed by the two tested isolates of MRSA at low concentrations range from (0.178-6.25 mg/ml).

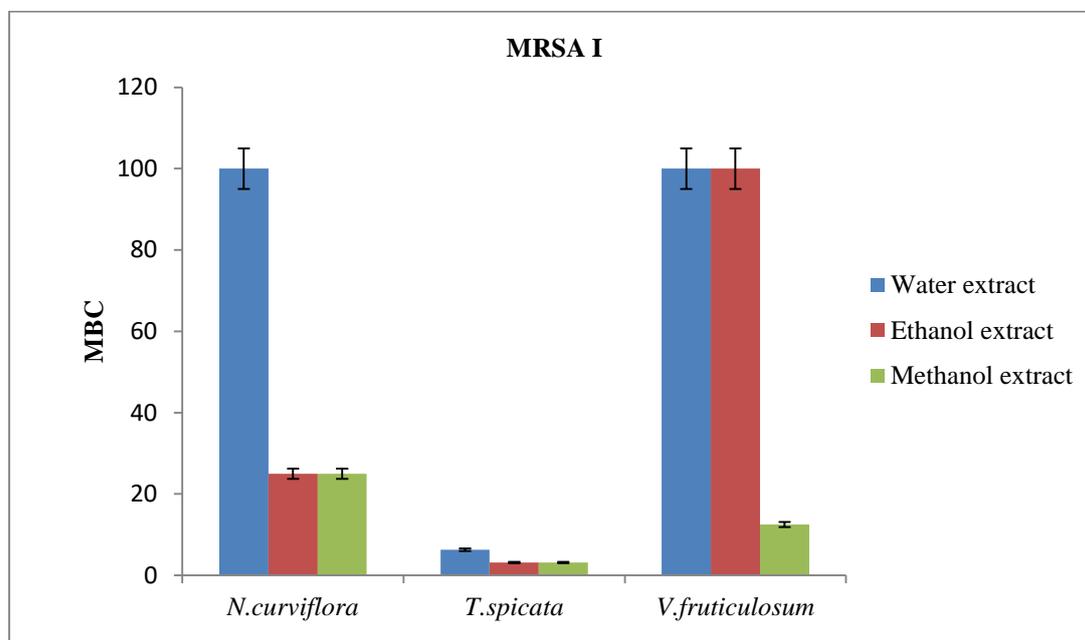


Figure 3. Antibacterial activity of *T. spicata*, *N. Curviflora* and *V. fruticulosum* against MRSA I; (MBC) minimum bacteriocidal concentration (mg/ml).

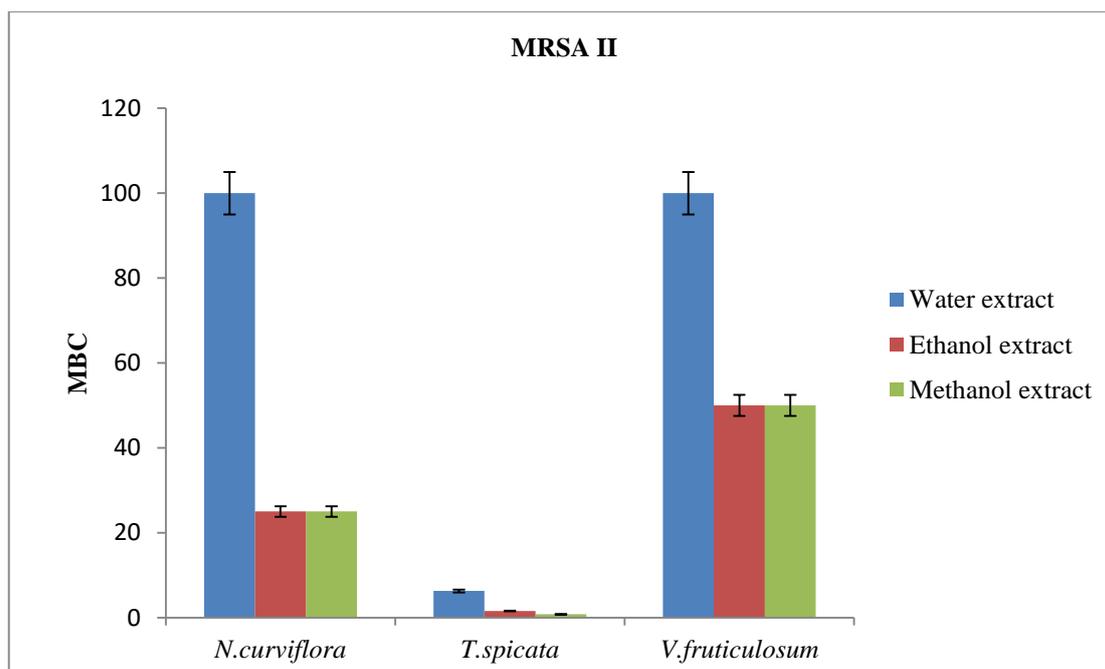


Figure 4. Antibacterial activity of *T. spicata*, *N. Curviflora* and *V. fruticulosum* against MRSAII; (MBC) minimum bacteriocidal concentration (mg/ml).

Discussion

In the past few decades, MRSA have caused a major problem with nosocomial infections throughout the world (Boucher and Corey, 2008). In the developed countries, fluoroquinolones (ciprofloxacin and ofloxacin) are recommended for serious infections associated with *Staphylococci* (Chambers, 1997), but vancomycin still remains the drug of choice for most MRSA-associated diseases (Smith et al., 1999). Searching for plants natural alternative to commonly used antimicrobial agents is increasing, particularly in Palestine. In the present study, we examined the effect of crude extracts (aqueous, ethanol and methanol) of three plant species (*T. spicata*, *N. curviflora* and *V. fruticulosum*) on two MRSA isolates. The two isolates were resistant to antibiotics, which are commonly used to treat MRSA including β - lactams and vancomycin. The finding of new antibacterial drugs is very important. The obtained results have proved that all *T. spicata* extracts, particularly alcoholic extracts had an inhibitory and bacteriocidal effects on both isolates even at low concentrations (0.195 to 6.25 mg/ml) of the studied crude extracts. These results were supported by previous studies, which focused on the essential oils of *T. spicata*. The essential oil and the main compounds, carvacrol and trans-caryophyllene, have been tested against different microbes which then showed strong antimycobacterial activity (Kilic, 2006). The essential oil of *T. spicata* plant was also tested against *S. aureus*, *Bacillus cereus*, *Salmonella typhimurium* and *E. coli*, and it was effective on *B. cereus* and *E. coli*, but no effect was recorded on *S. aureus* (Akin et al., 2010). Omar et al., (2013) studied the effect of crude extracts of *T. spicata* on different bacteria. Both water and ethanol extract have an inhibitory effect on *S. aureus*.

N. curviflora antimicrobial activity against MRSA can be explained as *Nepeta* species contain different types of essential oils such as nepetalactones. In India; *Nepeta* species were recorded to give antimicrobial activity against different bacteria including *S. aureus*, (Bisht et al., 2010). Other studies done on *N. crispa* in Iran and *N. nuda* in Turkey indicated that *Nepeta* essential oils could be a natural antibacterial agent (Mozaffarian, 1996; Alim et al., 2009). Our findings confirmed the findings of the previous studies, but the antimicrobial activity of the genus *Nepeta* have considered different species, which is *N. curviflora*. Alcoholic extracts of *N.*

curviflora had moderate Bacteriostatic and bacteriocidal effects on the examined MRSA isolates as they were effective at concentration equal to (12.5 mg/ml) and higher. Moreover; the results obtained from *V. fruticosum* crude extracts were somewhat similar to those obtained from other *Verbascum* species. For example, *V. mucronatum* and *V. olympicum* showed antibacterial activity against *S. aureus* (Kahraman et al., 2011). In another study, the methanol extracts of *Verbascum* species other than *V. fruticosum* have been found to be effective against gram positive bacteria, but no activity observed against gram negative bacteria by disc diffusion method (Dulger and Ugurlu, 2005a).

Conclusion

The present study showed that most of the prepared extracts have antibacterial activity. Among them *T. spicata* ethanol and methanol extracts are potentially good sources of antibacterial agents. This antibacterial activity of *T. spicata* crude extracts suggests the possibility of using these extracts in the treatment of infectious diseases caused by multi-drug resistant *S. aureus* bacteria. The antimicrobial activity of the crude extract may be attributed to a specific compound or a combination of compounds. The knowledge about the chemical profile of the extract helps in explaining the observed activity.

Recommendations

- Designing experiments for fractionation and isolation of the active compounds like alkaloids, flavonoids, coumarins, saponins and steroids, which are known to have antibacterial activity, are recommended.
- Further studies are necessary to explore the use of these plant extracts as safe alternatives to synthetic antimicrobial drugs.

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التأثير المضاد للبكتيريا لمستخلصات نباتية مختارة في المكورات العنقودية الذهبية

المقاومة للميثيسيلين

لبنى عبدالله¹ ، شروق اسماعيل²

قسم العلوم الحياتية والتقنيات الحيوية، كلية العلوم، جامعة النجاح الوطنية

alubna@najah.edu¹ , Shurooq.ismail@najah.edu²

الملخص

أجريت هذه الدراسة بناء على أهمية النباتات العشبية في علاج الأمراض. وكان الهدف الرئيسي للبحث هو فحص فعالية المستخلصات المائية والكحولية المأخوذة من ثلاث نباتات موجودة في فلسطين وهي (*Thymbra spicata* و *Nepeta curviflora* و *Verbascum fruticosum*) ضد عزلتين من المكورات العنقودية المقاومة للميثيسيلين. من خلال التجارب المستخدمة لقياس تأثير المستخلصات من البكتيريا *Well Diffusion Method* تم دراسة فعالية جميع المستخلصات. وبعد ذلك تم إيجاد التركيز المثبط الأدنى و التركيز القاتل الأدنى لكل المستخلصات الفعالة. وكان من الملاحظ أن المستخلصات الكحولية لجميع النباتات هي الأكثر فعالية مقارنة بالمستخلصات المائية. وأشارت النتائج إلى أن نبات (*Thymbra spicata*) هو الأكثر تأثيراً في المكورات العنقودية. حيث عملت مستخلصات هذا النبات على قتل عزليتي البكتيريا بتراكيز تتراوح بين (0,781 و 6,25) ملجم/مل. واستناداً إلى نتائج الدراسة فإننا نوصي بتحليل المكونات الكيميائية للنباتات المدروسة وإعادة دراسة تأثير كل مكون بشكل منفصل في المكورات العنقودية المقاومة للميثيسيلين، مما سيدعم الصناعات الدوائية ضد هذا النوع من البكتيريا.

الكلمات الدالة: البكتيريا العنقودية المقاومة للميثيسيلين، مضادات البكتيريا، مستخلصات نباتية.